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Evaluation of DNA/RNAshells for Room Temperature Nucleic Acids Storage

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Traditional nucleic acids preservation methods rely on maintaining samples in cold environments, which are costly to operate and time sensitive. Recent work validated that using room temperature for the storage of nucleic acids is possible if the samples are completely protected from water and oxygen. Here, we conducted accelerated aging and real-time degradation studies to evaluate the new technology DNAshell and RNAshell, which preserves DNA and RNA at room temperature, including the DNA and RNA yield, purity, and integrity. DNA and RNA solutions are dried in the presence of stabilizers in stainless steel minicapsules, then redissolved after different time points of heating and storing at room temperature. Results show that DNAshell and RNAshell ensure the safe storage of nucleic acids at room temperature for long periods of time, and that the quality of these nucleic acids is suitable for common downstream analysis.

Introduction

With the completion of the Human Genome Project, collection of DNA and RNA samples and related data for investigation and expansion of human genetic knowledge has increased.[1,2] Nucleic acids are valuable resources for clinical and scientific research, and also for forensic applications.[3] Through a combination of a person's genetic information and environmental factors and personal health history, scientists can attempt to undertake various studies that could identify genotype–phenotype relationships, achieve effective treatments of certain illnesses, and immensely improve the quality of human life.[4] The study of the effects of genetic factors in complex diseases needs to be supported by large-scale studies with high-quality human genetic materials. On the other hand, as of today, we could not design the clinical trials so comprehensively that the research would cover all valuable genes whose importance can be predicted. So the investigators must be able to preserve the nucleic acids for the long-term to enable future analyses.[5]

Nucleic acids, especially RNA, have proven to be problematic with regard to keeping their integrity over time in the context of high-quality diagnostic and prospective use of tissue in surgery.[2] Earlier studies have suggested that atmospheric water and oxygen could greatly affect the rate of degradation of solid-state DNA at room temperature.[6] RNA is more fragile than DNA and sensitive to ubiquitous ribonucleases (RNases).[7] Current methodologies used in the handling of nucleic acids samples rely on cold chain processes during collection, transportation, processing, and storage procedures, which are costly and time-sensitive. For biobanks and other preservation purposes, huge numbers of nucleic acids samples need to be stored, and classical storage in cold environments such as freezers appears cumbersome, costly and rife with inevitable risks of failure.[8] So it is useful to develop and apply an alternative method for storing samples at ambient temperature.

Commercial products that could preserve samples at room temperature, such as RNAlater reagent (Ambion),[9,10] as tissue and cell protection reagents are widely used nowadays: they can protect RNA *in situ* in unfrozen specimens at room temperature for at least a week. FTA cards will bind nucleic acids and inactivate ribonucleases, but the amounts that can be stored and retrieved are low and the storage time is limited.[11] GenTegra (GenTegra LLC), RNAstable (Biomatrica), and DNA/RNAshells (Imagene) could protect dehydrated nucleic acids at room temperature in the presence of hydrophilic additives.[12–14]

IMAGENE's technology is based on encapsulating purified and dehydrated nucleic acids in the airtight stainless steel DNAshell or RNAshell. The controlled (anhydrous and anoxic) atmosphere can protect the nucleic acids from deleterious factors (air, water, light) and allow preservation of nucleic acids at room temperature for long periods of time. This novel preservation method provides numerous advantages compared to traditional cryopreservation technologies,

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in particular in terms of the traceability, safety, and stability, operational and maintenance costs, transport, and distribution costs.

Clermont et al.[14] reported that the DNAshell could protect DNA samples for 100 years without detectable degradation through an accelerated aging study (76°C,50% humidity for 30 h), and that DNA samples stored in DNAshells at room temperature for 18 months showed no detectable degradation.

Mathay et al.[15] investigated the RNA preservation effects of GenTegra (GenTegra LLC), RNAstable (Biomatrica), and RNAshells (Imagene) at room temperature for 2 weeks, and showed that the RNA quality was guaranteed when preserved under those conditions, which is helpful for shipment and short-term storage at RT. But RNA long-term stability under those storage conditions was not assessed.

The degradation rate of the nucleic acids depends on temperature and has been shown to obey the Arrhenius Law.[16,17] Here we conducted accelerated aging (60°C) and real-time degradation studies to evaluate the long-term preservation quality of DNAshell and RNAshell, including the DNA and RNA yield, purity, and integrity. We showed that DNAshell and RNAshell allow for DNA and RNA long-term preservation at room temperature, and the quality is suitable for common downstream analyses.

Materials and Methods

Sample preparation

DNA was extracted from human whole blood cells using an Automated Puregene (Qiagen) instrument according to the manufacturer's instructions, and dissolved in TE buffer (Magen). RNA was extracted from BL-2 (Burkitt lymphoma) cell lines, and the cells pellets (1×10^7) were resuspended in 1 mL of lysis buffer (4 M guanidine thiocyanate, 1% b-mercaptoethanol, 40 mM sodium acetate, 0.17% sarkosyl) and 10% sodium acetate 2 M pH 4. RNA was extracted twice by 1.2 volume of phenol:chloroform:isoamyl alcohol (250:49:1) and then precipitated by adding 1 volume of isopropyl alcohol and 0.1 volume of sodium acetate (2 M, pH 4) to the aqueous phase. The RNA pellet was washed with 70% ethanol and resuspended in RNAse-free water.

The same original extracted DNA and RNA samples were aliquoted to equal volumes into DNA/RNAshells and corresponding EP-tubes. The DNA and RNA solutions were mixed with Imagene proprietary stabilizer and deposited in the completely airtight stainless steel capsules, which contain a glass insert. The DNA (3 μg) and RNA (1 μg) were vacuum-dried prior to laser-welding the capsules inside a dry gloves box. In this experiment, DNAshells and RNAshells were provided by Imagene and transported to BGI at room temperature within 5 days. Samples without any protection were transported on dry ice and stored at −80°C immediately upon reception. The quantity and quality of all the samples protected by shells and without any protection were analyzed in BGI-Shenzhen, respectively, before the tests (both accelerated aging studies and real-time degradation studies), and the detection time point was set as T_0 according to the study design shown in Figure 1.

Accelerated aging studies

In order to evaluate the quality of DNA and RNA rapidly, the accelerated aging experiment was performed at 60°C and different incubation times. The estimation of the corresponding time at room temperature was calculated by extrapolation using the Arrhenius model. Indeed the degradation rate of the nucleic acids depends on temperature and has been shown in previous works to obey the Arrhenius Law. The experiments reported here having been run in the same conditions, we may assume that the same temperature dependence occurs (see below).

After the heating incubation, the DNA and RNA were recovered simply by adding buffer. The yield volumes were

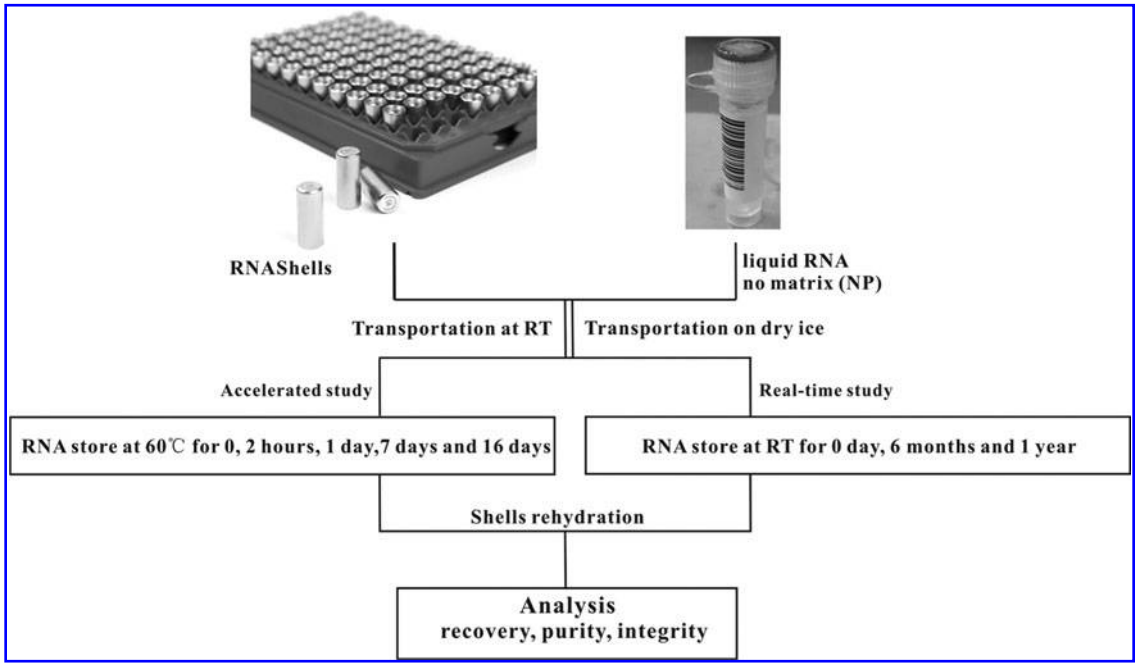

FIG. 1. Pilot study workflow.

calculated according to the target concentration. DNA and RNA were dissolved separately in 30 μL TE buffer and 20 μL RNase free water, respectively. After 5 to 10 minutes of rehydration, DNA and RNA solutions can be used for further analysis.

The degradation rates of encapsulated DNA and RNA, at the temperatures of interest (25°C and 60°C), can be drawn respectively from Reference 17, Figure 10 and Reference 16, Figure 3. From that information, we calculated the ratios $k_{60°C}/k_{25°C}$ ($k_{60°C}/k_{25°C} \approx 160$ for RNA, $k60_{°C}/k25_{°C} \approx 630$ for DNA). Such a ratio gives the time (t_{60}) necessary to incubate the samples at 60°C to mimic a given storage time (t_{25}) at 25°C. Indeed the number of cuts/nucleotide at 25°C (cn_{25}) is given by

$$cn_{25} = k_{25}.t_{25}$$

and the number of cuts/nucleotide at 60°C (cn_{60}) is given by

$$cn_{60} = k_{60}.t_{60}$$

The aim of the calculation is to determine the time t_{60} where the number of cuts at 60°C will be equal to the number of cuts produced by a given storage time (t_{25}) at 25°C. So we have:

$$cn_{25} = cn_{60} = k_{25}.t_{25} = k_{60}.t_{60}$$

and consequently:

$$t_{25}/t_{60} = k_{60}/k_{25}$$

DNA samples were stored for 0 h, 4 h, 39 h, 91 h, and 8 days under various conditions. Storage in DNAshells and storage in elution buffer without protection (Non-protected NP) at 60°C are equivalent to approximately 0 day, 100 days, 3 years, 7 years, and 14 years storage at room temperature, respectively.[17]

RNA samples were stored for 0 h, 2 h, 1 day, 7 days, and 16 days under various conditions. Storage in RNAshells and storage in RNase free water without protection (NP, non-protected) at 60°C are equivalent to approximately 0 day, 14 days, 100 days, 3 years, and 7 years storage at room temperature.[16]

Real-time degradation study (storage at room temperature)

DNAshells and RNAshells were stored at room temperature (20 ~ 25°C) after being transported to BGI. The quality of the DNA and RNA were analyzed after 6 months and 1 year (DNA for 14 months) preservation.

Quantitative and quality analysis

The DNA samples were analyzed by a Qubit fluorimeter (QUBIT Q32857), NanoDrop (Thermo Scientific, ND-8000), and agarose gel electrophoresis (AGE), to measure yield, purity, and integrity after rehydration. The integrity and yield of RNA samples were quantified by Agilent 2100; NanoDrop was also applied to detect the RNA purity.

DNA yield, purity, and integrity after rehydration

After thorough mixing, 1 μL samples were measured using the Quant-iT™ ds DNA Broad Range kit according to Qubit Fluorometer protocol, to determine the rate of yield from DNAshells. The A260/A280 and A260/A230 ratios of the DNA samples were analyzed by the NanoDrop system following the standard protocol for DNA purity assessment. Finally the integrity and the size of DNA were checked by AGE (120 v, 30 min) in 1% agarose gels stained with ethidium bromide and photographed with a Tanon 1600 system.

RNA yield, purity, and integrity after rehydration

RNA yield and integrity were determined by Bioanalyzer (Agilent Technologies, G2939A), which is a common tool allowing separation with high resolution as well as quantification with high accuracy and is suitable for reliable quality control of RNA. RNA purity was detected by NanoDrop.

Statistical analyses

Data were analyzed using Graphpad Prism software. Results are presented as means ± standard deviation (SD), with $N = 3$. The significance was determined by the test and p value < 0.05 was regarded as a significant difference.

Results

DNA quantity and quality analysis

To test the quantity and quality of DNA (3 μg) stored in DNAshells and in a non-protected condition, we first measured the samples by the Qubit fluorometer, and verified that the yield of all DNAshells (stored at 60°C for 4 h, 39 h, 91 h, and 8 days) were higher than 2.74 μg, and that no statistically significant differences were detected when compared to control samples. However, without any protection, DNA yields had statistically significant differences after heating for 39 h at 60°C and compared with T_0 (Supplementary Table S1). Next, we utilized NanoDrop to examine the purity of DNA by measuring the A260/A280 ratio. The purity of DNAshells was affected significantly by the accelerated aging experiments after heating for 8 days at 60°C (Table 1).

In addition, we checked the integrity of DNA by AGE. In the DNA accelerated test, DNA in the shells showed a slight degradation as time went on; but without protection, DNA samples were degraded completely when they were stored for 39 hours at 60°C (Fig. 2).

In order to reflect the preservation situation more accurately, some of the DNAshells were stored at room temperature for 14 months prior to analysis. We measured the yield and purity (A260/A280 ratio, 260/230 ratio) of the DNA samples at different time points such as 6 months and 14 months, and compared those data with the samples protected by DNAshells and detected at T_0. Results indicated that the DNAshell could maintain the DNA samples in good condition, and there was little difference compared to control samples (Table 1). We checked the integrity of DNA by AGE, for the samples protected by DNAshells at room temperature, with no obvious degradation can be detected after 14 months. However, without protection DNA samples degraded seriously when were stored for 9 days at RT (Supplementary Fig. S1 and Fig. S2; supplementary material is available in the online article at www.liebertonline/bio).

RNA quantity and quality analysis

In the RNA accelerated aging test, RNA yield from RNAshells detected by Agilent 2100 ranged from 1.09 μg to

Table 1. DNA Quality Controls (Mean ± SD)

Type	*Time*	*Temp.*	*Yield (μg)*	*Purity*	
				260/280	*260/230*
Non-protected DNA samples	0 day	−80°C	2.72 ± 0.16	1.85 ± 0.01	1.89 ± 0.07
	14 months		2.59 ± 0.10	1.84 ± 0.01	1.51 ± 0.07
DNAshells	0 day	RT	3.13 ± 0.40	1.89 ± 0.03	1.64 ± 0.04
	6 months		3.25 ± 0.48	1.88 ± 0.04	1.53 ± 0.05
	14 months		3.32 ± 0.43	1.84 ± 0.01	1.45 ± 0.02
	0 hour	60°C	3.13 ± 0.040	1.89 ± 0.03	1.64 ± 0.04
	4 hours		2.74 ± 0.23	1.88 ± 0.03	1.54 ± 0.04
	39 hours		2.82 ± 0.39	1.82 ± 0.05	1.59 ± 0.21
	91 hours		2.89 ± 0.55	1.82 ± 0.05	1.43 ± 0.05
	8 days		2.88 ± 0.34	1.76 ± 0.03	1.58 ± 0.07

RT, Room temperature; Temp, temperature; the quantity and quality of non-protected DNA samples and DNA stored in DNAshells were detected by Qubit fluorometer and NanoDrop, respectively.

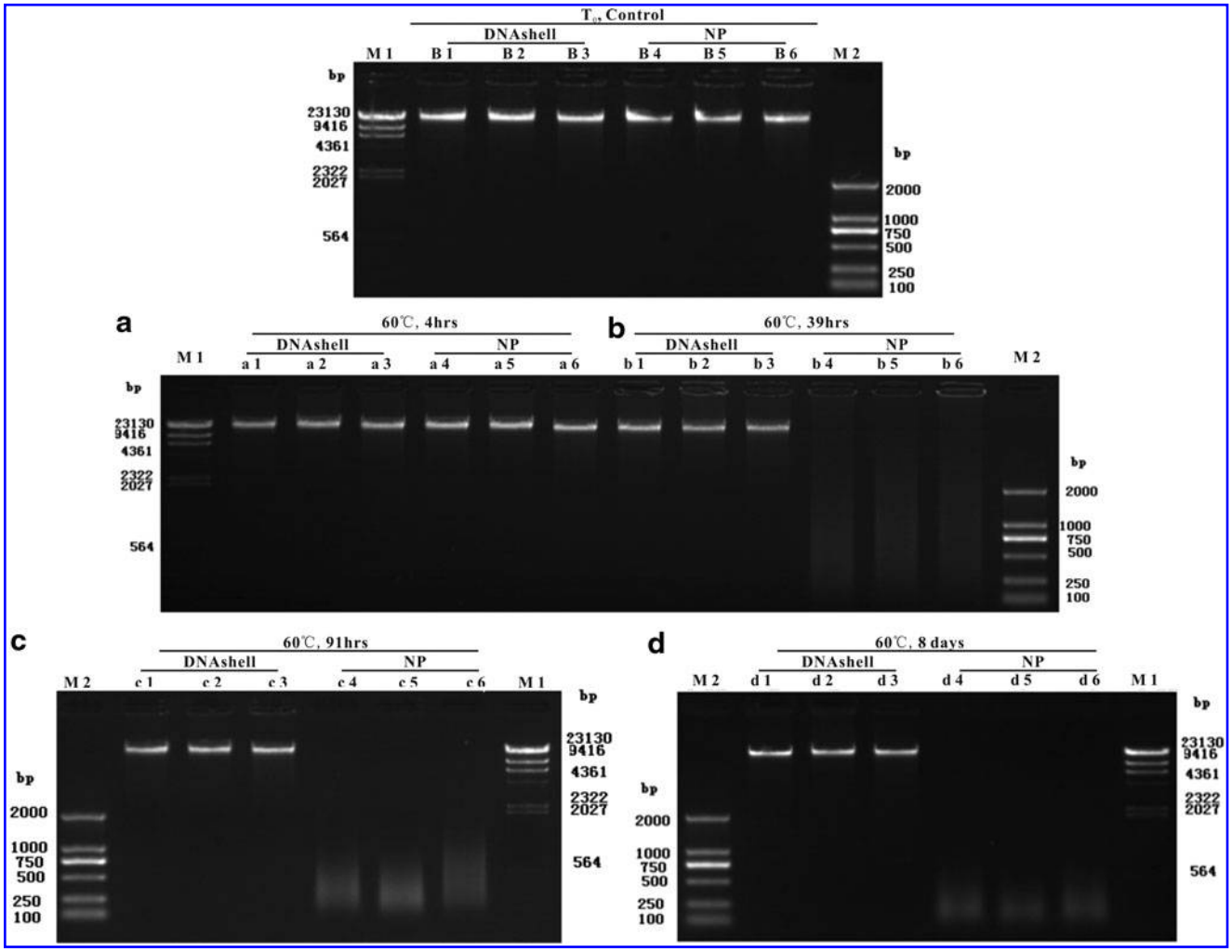

FIG. 2. DNA integrity measured by agarose gel electrophoresis in accelerated test. DNA integrity was measured by AGE, the control group is shown in image. Control, B1-B3, and B4-B6 represent the DNA stored in DNAshell and non-protected condition at T0 of the accelerated aging study. All experimental samples were loaded on four gels according to the time course shown in images **a, b, c, and d.** *Lanes 1–3*: protected by DNAshell (a1–a3, b1–b3, c1–c3, d1–d3), *lanes 4 and 5*: non-protected samples (a4–a6, b4–b6, c4–c6, d4–d6). M1 and M2 display DNA ladder, M1:λ-Hind III digest (Takara), M2: D2000 (Tiangen).

1.42 μg and showed no downward trend. With no protection, RNA yield sharply decreased in the 60°C condition, and a significant difference compared to the control (characterized by BGI-Shenzhen at T_0) was observed after 1 day of heating at 60°C (Fig. 3A and Supplementary Table S2).

Analysis of RNA integrity and purity served as tools to determine RNAshells storage characteristics. The RNA Integrity Number (RIN) and 28S/18S values are both factors that are indicative of the RNA integrity. In the experiment, the RIN values and 28S/18S values were tested by Agilent 2100. All RNA in RNAshells under different storage times showed some reduction in RIN values, although they never dropped below 7. The largest drop was observed after heating for 7 days (Fig. 3B-a). The RIN values of the RNA without any protection are much less than 7, showing that the RNA integrity was substantially compromised. 28S/18S values showed a similar situation (Fig. 3B-b).

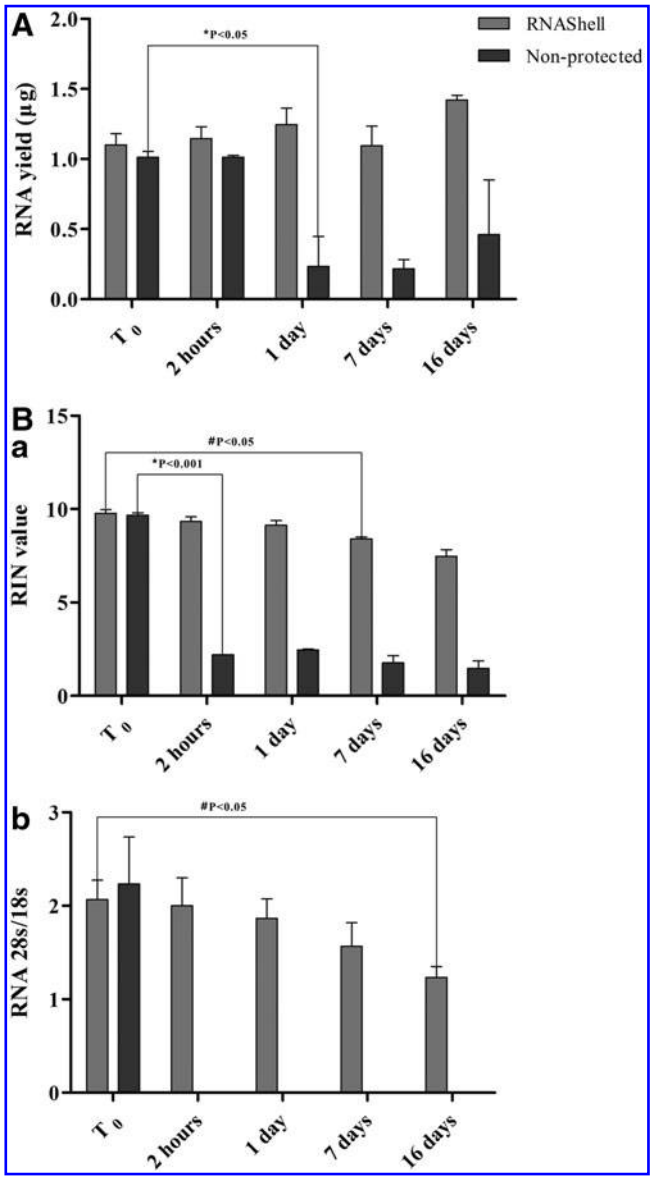

FIG. 3. RNA recovery and RNA integrity measured by bioanalyzer. Each storage condition is shown for protected by RNAshell (■) and non-protected (■, RNA in solution, no matrix). The samples provided by Imagene, Non-protected samples analyzed by BGI-Shenzhen at T0 of the accelerated aging study were used as controls; *bars* correspond to the standard error of mean. **(A)** RNA recovery, for RNAshells and non-protected samples, RNA was quantified by bioanalyzer and data are shown as mean recovery percentages ± SD ($N=3$),*$p=0.0229<0.05$. **(B)** RNA integrity was measured by RNA RIN **(a)** and 28S/18S **(b)**. Data are shown as means ± SD ($N=3$); **a**, $^{\#}p=0.0013<0.05$, *$p<0.001$; **b**, $^{\#}p=0.0285<0.05$.

For the samples protected by RNAshells and stored at room temperature, we measured the RNA yield, integrity (RIN, 28s/18s) and purity at different time points including 0 days, 6 months, and 1 year (Table 2). The results showed that RNA quality changed little after storing for 1 year at RT, and there was no significant difference observed. And the RNA integrity when stored at −80°C (Fig. 4-a) was as good as the samples protected by RNAshell (Fig. 4-b, RT), and both RIN values were greater than 9.5.

Discussion

In this pilot study, our objective was to assess the long-term preservation capabilities of DNAshell and RNAshell through an accelerated aging study and real-time degradation study.

The accelerated aging study, based on the previous observation that the degradation rates of both RNA and DNA obeyed the Arrhenius Law, was conducted by raising the temperature to 60°C to simulate longer storage at ambient temperature. We evaluated the DNA and RNA stabilities for the equivalent of approximately 14 and 7 years storage at room temperature, respectively, including the DNA/RNA yield, purity, and integrity. These results can be used in planning other biobanking applications such as long-term DNA and RNA storage.

The results showed that regardless of the use of protection (or not), the DNA and RNA degraded over time. But DNAshells and RNAshells can protect the DNA and RNA samples efficiently for a long time compared with non-protected ones. DNA is more stable and its degradation rate is much lower than that of RNA.

After accelerated aging for 8 days (equivalent to approximately 14 years storage at room temperature), the DNA stored in DNAshell was still recoverable at a high rate, the purity remained in the normal value range, and the integrity analysis by AGE showed only a slight degradation; but the DNA samples without any protection showed a sharp degradation. For those samples stored in DNAshells for 6 months or 14 months at room temperature, the yield and purity (A260/A280 ratio) changed little, and there was no detectable degradation observed based on AGE analysis.

Fluctuations of the RNA yield were seen in the RNAshell accelerated aging study, but all of the yields were higher than 1 μg. These results may be caused by instrumental error (analytical variability), and it has been reported that RNA recovery could be greater than 100% for RNA stored at RT.[12] As a rule, RNA samples with RIN values in the range of 7.0 to 10.0 are identified as good quality and it is suggested that RNA quality could fulfill the requirement of highly demanding gene array assays with RIN values > 7 (www.asterand.com/Asterand/human_tissues/Asterand_RIN.pdf). Although the RIN value showed s significant difference

Table 2. RNA Quality Controls (Mean ± SD)

Type	Time	Temp.	Yield (μg)	Integrity		Purity	
				RIN	28s/18s	260/280	260/230
Non-protected RNA samples	0 day	−80°C	1.01 ± 0.07	9.67 ± 0.25	2.23 ± 0.50	N/A	N/A
	6 months		0.86 ± 0.09	8.73 ± 0.25	1.20 ± 0.10	N/A	N/A
	1 year		1.02 ± 0.12	9.77 ± 0.21	1.90 ± 0.00	1.75 ± 0.01	2.21 ± 0.08
RNAshells	0 day	RT	1.10 ± 0.08	9.77 ± 0.21	2.07 ± 0.21	2.04 ± 0.03	1.71 ± 0.06
	6 months		1.03 ± 0.03	9.53 ± 0.12	1.87 ± 0.15	2.00 ± 0.07	1.66 ± 0.04
	1 year		1.05 ± 0.13	9.53 ± 0.12	1.90 ± 0.26	2.05 ± 0.02	1.67 ± 0.09
	0 hour	60°C	1.10 ± 0.08	9.77 ± 0.21	2.07 ± 0.21	2.04 ± 0.03	1.71 ± 0.06
	2 hours		1.15 ± 0.08	9.33 ± 0.25	2.00 ± 0.30	2.03 ± 0.02	1.89 ± 0.04
	1 day		1.25 ± 0.12	9.13 ± 0.25	1.87 ± 0.21	2.03 ± 0.09	1.78 ± 0.23
	7 days		1.09 ± 0.14	8.40 ± 0.10	1.57 ± 0.25	2.09 ± 0.04	1.80 ± 0.03
	16 days		1.42 ± 0.03	7.47 ± 0.35	1.23 ± 0.12	2.07 ± 0.03	1.74 ± 0.02

NA, Not available, RT, Room temperature; Temp, temperature. The quantity, integrity. and purity of non-protected RNA samples and RNA stored in RNAShells were detected by Agilent 2100 and NanoDrop, respectively.

on the 7th day compared to the control, the RIN value was still greater than 7.0. In addition, the RNA 28S/18S downward a trend and is similar to the RIN value, and showed a good quality and integrity. There was little variability in the yield, RIN value and purity for those samples stored in RNAshells for 6 month or 1 year at room temperature.

Our nucleic acids have been prepared using standard techniques and are of standard good quality. One question is whether nucleic acids of suboptimal quality will also be protected to the same degree. Data reported by Clermont et al.[14] showed that, in DNAshells, partially degraded DNA did not degrade faster than high quality DNA. It is also well known that the stability of nucleic acids can widely vary according to their purity. This is probably due to the contaminants co-purifying with nucleic acids. A study was conducted with the aim to correlate the presence of contaminants and DNA stability in DNAshell, by extracting DNA from various origins and using several extraction procedures. Variability in stability was seen, but this did not impact significantly on the storage quality of the samples. In particular, in all the samples, no evidence of degradation could be found after a simulated 25–100 year storage period at room temperature in DNAshells.[18]

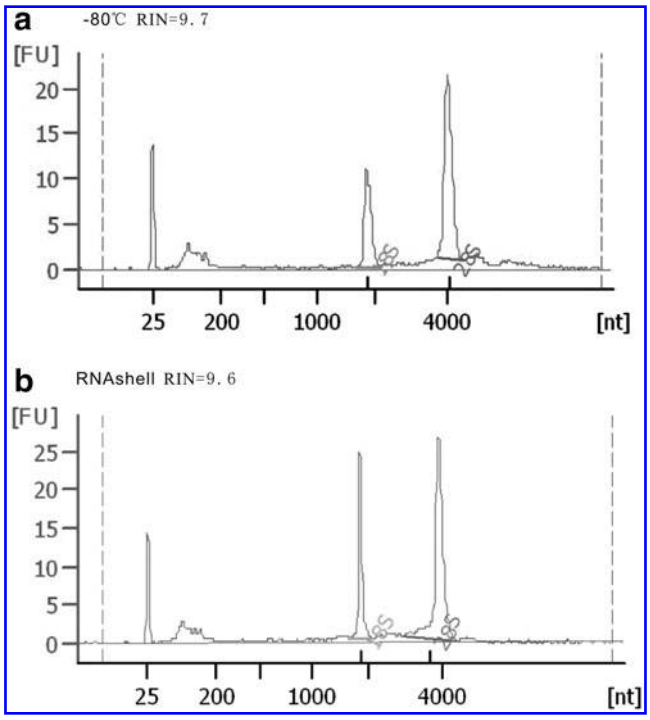

FIG. 4. RIN values of samples stored at −80°C (**a**, control) and protected by RNAshell (**b**) for 1 year.

The question is of particular importance for nucleic acids extracted from clinical biospecimens that can be degraded before storage. This degradation is generally due to the quality of the tissues, but in most cases this will not lead to higher contamination after extraction, and consequently the rate of degradation in most cases should not be faster for these samples.

More specifically, for RNA it has been observed that the RNA species (mRNA, rRNA), the origin (fungal, bacterial, or mammalian), the extraction/ purification technique (acidic phenol, Trizol, silica adsorption/washing/elution or HPLC purification), the initial quality (RIN = 9 or 7) and the resuspension buffer (Tris alone, Tris EDTA or water), the measured degradation rates exhibited no variation.[16]

We have demonstrated that the quality of DNA/RNA samples was as good as the original samples after transportation to BGI-Shenzhen at RT for about 1 week. We also show that the DNA and RNA quality was still high after storage simulating about 14 years and 7 years at RT, respectively. These results indicated that DNA/RNAshells allow for DNA/RNA short-term and long-period preservation at room temperature and are suitable for common downstream analysis. These findings suggest that the shells could be used to transport solid nucleic acids at RT, and avoid the risks of cold chain transportation. So shells could help to reduce the cost of effectively storing dried nucleic acids at RT compared to freezer storage, which, beyond being more expensive to power and maintain, are submitted to risks of power failure or natural catastrophes. Other advantages of the use of minicapsules for the large DNA and RNA banks we are currently building is the fact that the samples are packed automatically into 2-D barcoded minicapsules, which improves traceability level, and simplifies storage management.

In conclusion, our study demonstrated that the storage vessels DNAshell and RNAshell could preserve safely and cost-effectively high quality DNA and RNA for long periods of time at room temperature.

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Author Disclosure Statement

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